PAGE4 Is a Cytoplasmic Protein That Is Expressed in Normal Prostate and in Prostate Cancers

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Abstract

***PAGE4* is an X chromosome-linked cancer-testis antigen that was identified by expressed sequence tags database mining and a functional genomic approach. *PAGE4* is preferentially expressed in normal male and female reproductive tissues and also in a variety of cancers including prostate. In the present study, we have used *in situ* hybridization to show that *PAGE4* mRNA is expressed only in the epithelial cells of normal and prostate-cancer specimens. Analysis of the protein product encoded by the *PAGE4* mRNA reveals that it encodes a *M*, 16,000 protein and is detected in tissue extracts from both normal prostate and prostate cancer. Cell fractionation analysis of PAGE4 protein indicates that PAGE4 is localized in the cytoplasm of the cell. Furthermore, cDNA microarray analysis indicates that the expression of *lipoprotein lipase*, a gene frequently deleted in prostate cancer, is down-regulated in a cell line that expresses *PAGE4*.**

Introduction

Prostate cancer is a major public health problem and the second leading cause of death for men in the United States. About one in five men in the United States will develop prostate cancer during their lifetime. Despite its distinction as the most frequently diagnosed noncutaneous cancer, little is known about the causes of this disease largely because of the cellular heterogeneity of the prostate and the lack of systematic analysis of the genes expressed in this tissue. Completion of the human genome project and the technological advancement in biomedical research has enabled researchers to identify and to systematically analyze new genes that could be used as targets for cancer therapy or that could be involved in the multistep process of cancer. Many different methods are currently being used to identify tissue- or cancer-specific genes. Our laboratory is interested in identifying genes by using the EST[3] database, and developed a computer-based screening strategy to generate clusters of ESTs that are specifically expressed in normal prostate and/or prostate cancer but not in essential normal tissues (1). We then used experimental approaches to verify these predictions. Using this approach, we identified *PAGE4*, a new gene expressed in normal prostate and testis and highly expressed in prostate and uterine cancers (2).

In this report, we analyzed *PAGE4* mRNA expression by *in situ* hybridization using several prostate cancer samples. We also report here the characterization and subcellular localization of the protein encoded by *PAGE4* mRNA. Using cell fractionation and immunofluorescence analysis we demonstrate that the *M*, 16,000 protein product of *PAGE4* is localized in the cytoplasm of the cell. In addition, using cDNA microarray analysis, we report that the expression of *LPL*, a gene involved in lipid metabolism, is down-regulated in cells expressing *PAGE4*.

Materials and Methods

Primers. Nucleotide sequences of the primers used in this study were: forward CP1 (5′-AAGAGGAATTCGCAGCGATGAGTGCACGA-3′), and reverse CP2 (5′-GCACTGAATTCTCAGCCATGTGTGTAGCT-′); forward CR1 (5′-AAGAGCATATGAGTGCACGAAGTGAGTCA-′), and reverse CR2 (5′-CACTCTCGAGTGGCTGCCCATCTCCTGCTTC-3prime]); and forward LU1 (5′-TGCATGTGTGTTGTCCTTCAG-3′), and reverse LU2 (5′-GAGTTCCCAGTGCTATGTGCTGCT-3′). All of the primers were synthesized by Genosys (The Woodlands, TX).

Constructs. The pCI-*PAGE4* plasmid was constructed from the pLZR-SpBMN-Z vector (3) replacing the *lacZ* gene with the *PAGE4* cDNA. The DNA fragment encoding the open reading frame of *PAGE4* was amplified from the *PAGE4* cDNA using primer pairs CP1 and CP2. The PCR product was gel purified, digested with *Eco*RI, and ligated into an *Eco*RI-digested pLZR-SpBMN-Z vector. The pET-*PAGE4* plasmid was constructed using the pET23a plasmid (Novagen, Madison, WI). The DNA fragment encoding the open reading frame of *PAGE4* was amplified from the *PAGE4* cDNA using primer pairs CR1 and CR2. The PCR product was gel purified, digested with *Nde*I and *Xho*I, and ligated into a *Nde*I-*Xho*I-digested pET23a vector.

The pBS-*PAGE4* plasmid was constructed using the pBluescript II SK (+) plasmid (Stratagene, La Jolla, CA). The pBS-*PAGE4* plasmid contains the nucleotides 1–442 of the *PAGE4* transcript.

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[3] The abbreviations used are: EST, expressed sequence tag; CT, cancer-testis; Dlk1, δ-like homologue *Drosophila*; IGFbp-2, insulin-like growth factor binding protein 2; LPL, lipoprotein lipase; RT-PCR, reverse transcription-PCR; NCI, National Cancer Institute; CCR, Clinical Cancer Research.

Cell Lines and Culture. NIH3T3 and 293T cells were maintained at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (Quality Biological, Inc., Gaithersburg, MD), 2 mM L-glutamine, and 1 mM penicillin-streptomycin. PC3 cells were maintained at 37°C in 5% CO_2 in RPMI medium supplemented with 10% fetal bovine serum (Quality Biological), 2 mM L-glutamine, 1 mM sodium pyruvate and penicillin-streptomycin.

Packaging Cells Transfection and Target Cells Infection. The amphotropic 293T packaging line was transfected by a calcium-phosphate/chloroquine method described previously (3). Culture supernatants containing viral particles were collected 48 h after transfection, filtered with a 0.22 μm filter unit (Millipore, Bedford, MA), and used to infect the target cells. For stable infections, cells were plated in dishes of 100 mm at low density (300 cells/dish) and medium density (1000 cells/dish) 48 h after the infection. Three days after the infection, NIH3T3 cells were selected with 2.5 μg/ml puromycin, and PC3 cells were selected with 0.5 μg/ml puromycin. After 15 days, individual clones were picked and grown in the absence of puromycin.

Comparative cDNA Microarray Analysis. The protocol used for microarray analysis was designed by the Microarray Core Facility at NCI and can be found on the NCI/CCR microarray homepage.[4] Mm-OC-6.1p mouse array chips were obtained from the NCI microarray core facility. After hybridization, arrays were scanned using an Axon GenePix 4000 scanner and processed using the GenePix software. The results were analyzed using tools found on the NCI/CCR microarray homepage.

RNA Isolation for Microarray Analysis. Total RNA was isolated from the clones Infected with pCI-*PAGE4*, pLZR-SpBMN-Z, and the empty vector. The RNA was prepared by using TRIzol Reagent according to the manufacturer's instructions (Life Technologies, Inc., Rockville, MD) with the following modifications: after the chloroform addition and the phase separation, the aqueous phase was used in a second round of purification using the Rneasy Maxi Kit (Qiagen, Chatsworth, CA) as recommended by the manufacturer.

mRNA Extraction and Northern Analysis. mRNA from each cell line was isolated using a FastTrack kit (Invitrogen, Carlsbad, CA). Two μg mRNA per lane were electrophoresed under denaturing conditions and subsequently transferred to a nylon membrane according to established procedure. The *PAGE4* cDNA fragment was labeled with ^{32}P by random primer extension (Lofstrand Labs Ltd., Gaithersburg, MD). Hybridization was performed as described previously (4).

Dot Blot Analysis of Matched Tumor/Normal Expression Array. A membrane with 68 separate samples of cDNA synthesized from human tumors and corresponding normal tissue from the same individual (Clontech, Palo Alto, CA) was hybridized with a ^{32}P-labeled *PAGE4* cDNA fragment (Lofstrand Labs Ltd.). Hybridization conditions were described previously (4).

RT-PCR. For the analysis of LPL expression on PC3 and prostate mRNA, RT-PCR was performed as described previously (4) using primer pair LU1 and LU2.

***In Situ* Hybridization.** The paraffin-embedded prostate tissue sections were deparaffinized by placing the slides over a slide warmer at 65°C for 1 h. The slides were then rinsed in two changes of xylene for 5 min each and air-dried. They were then rinsed in two changes of absolute alcohol for 5 min and air-dried. Biotinylated probes were prepared using *PAGE4* (442 bp) and U6 (250 bp) cDNA cloned in pBluescript II (+) plasmid. Biotinylated pBluescript II (+) without any insert was used as negative control. Probes were labeled using the BioNick Labeling System kit (Life Technologies, Inc., Rockville, MD) according to the manufacturer' s instructions. The probes were incubated at 16°C for 3 h. The unincorporated nucleotides from the labeled DNA probe were removed by three ethanol precipitations. Slides were hybridized using the *In Situ* Hybridization and Detection system (Life Technologies, Inc.) according to the manufacturer' s instructions. The slides were counterstained using 0.2% Light Green staln, rinsed through a series of alcohol grades, and mounted in Cytoseal. Microscopic evaluation (brightfield) was performed using a Nikon Eclipse 800 microscope (5).

Preparation of Cell Extracts. Protein extracts from different cell lines were prepared as described previously (6). Briefly, about 5×10^6 growing cells (80% confluent) from each respective cell line were harvested and resuspended in 1× RIPA buffer containing proteinase inhibitors [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin]. The extracts were rotated for 30 min at 4°C and clarified by centrifugation. Protein concentrations were determined by using the Coomassie Plus Protein Assay reagent according to the manufacturer's instructions (Pierce, Rockport, IL).

Protein extracts from normal prostate and prostate cancer tissue were prepared by grinding 0.5 g of tissue frozen at −80°C into a fine powder using a cold mortar and pestle. The powdered tissue was collected, resuspended in 1× RIPA buffer, and sonicated briefly and clarified by centrifugation.

Nuclear, membrane, and cytoplasmic extracts from NIH3T3 and PC3 cells, infected with *PAGE4*, were prepared based on published protocols (7, 8).

Preparation of Recombinant PAGE4 Protein. The plasmid pET-*PAGE4* encodes amino acids 1 to 102 of *PAGE4* with six histidines at the COOH terminus encoded by the vector to facilitate purification of the protein. The recombinant PAGE4 protein was then expressed in *Escherichia coli* and purified using Ni^{2+}NTA agarose matrix following the supplier's instructions (Qiagen Inc.).

Production of Polyclonal Anti-PAGE4 Antibodies in Rabbits and Purification of IgG from Antisera. Purified PAGE4 protein was diluted to 0.5 mg/ml and injected into rabbits with complete Freund's adjuvant for the first immunization, and with incomplete Freund's adjuvant for subsequent immunizations. For PAGE4 antipeptide antibody, a peptide of 15 amino acids (amino acids 46–63) was synthesized and then injected into rabbits as described above. Sera

[4] Internet address: http://nciarray.nci.nih.gov/.

were collected after the fourth, fifth, and sixth immunizations and titrated by ELISA against the purified recombinant PAGE4 protein. Total IgG was then purified with immobilized protein A (Pierce) matrix following the supplier's instructions.

For tissue analysis, the *PAGE4* antisera were further purified by using immobilized *E. coli* lysate kit (Pierce) according to the manufacturer's instructions. The *PAGE4*-peptide antisera were further affinity-purified by using a HiTrap *N*-hydroxy-succinimide-activated column coupled with the recombinant *PAGE4*, according to the manufacturer's instructions.

Western Blot Analysis. Ten μg of protein extract from cell lines and 80 μg from tissues were run on a 16.5% Tris-Tricine gel (BIO-RAD, Hercules, CA) and transferred to a 0.2 μm Immun-Blot polyvinylidine difluoride membrane (BIO-RAD) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol (pH 8.3)] at 4°C for 2 h at 50 V. Filters were probed with 10 μg/ml protein A-purified anti-*PAGE4* antiserum or 1 μg/ml affinity column-purified anti-*PAGE4*-peptide antiserum, and their respective signals were detected using a chemiluminescence Western blotting kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN).

Immunofluorescence Analysis. Immunofluorescence analysis was performed as previously described (9). Briefly, 293T and NIH3T3 cells, transfected with pCI-*PAGE4* plasmid, were grown in 35-mm dishes. Cells were then fixed in 3.7% formaldehyde in PBS for 15 min at 23°C and then washed in PBS. All of the subsequent incubations (at 23°C) included 0.1% saponin (Sigma Chemical Co., St. Louis, MO) and 1% BSA in PBS (BSA-sap-PBS). Fixed cells were first incubated in BSA-sap-PBS for 30 min and then incubated with affinity-purified anti-PAGE4 antibodies (10 μg/ml) for 30 min. After washing in PBS, the cells were incubated with affinity-purified goat antirabbit IgG (H+L) conjugated to rhodamine (25 μg/ml) in BSA-PBS for 30 min. After washing, cells were fixed in 3.7% formaldehyde and mounted under coverslips in buffered glycerol. Microscopic evaluation of cells expressing PAGE4 protein was performed by direct observation of cells using a Zeiss Axiplan2 fluorescent microscope equipped with rhodamine filters, and images were captured using a Dage 300 cooled-chip charge coupled device camera.

Results

Expression of *PAGE4* in Various Cancers. By using Northern blot and RT-PCR analysis, we previously reported that *PAGE4* is expressed in normal prostate, prostate cancer, and female reproductive tissues (2). To determine whether *PAGE4* is expressed in other types of cancer, we conducted a cDNA dot blot analysis using a cancer-profiling array (Clontech). This array contains samples from 68 different cancers and their corresponding normal tissues. As shown in Fig. 1, among the 68 different samples from human normal and cancer tissues, *PAGE4* was detected in all three of the normal prostate samples (D11, 12, and 13) and prostate cancer samples (E11, 12, and 13). Moreover, *PAGE4* was found in one normal cervix sample and cervical cancer sample (G14 and H14, respectively). *PAGE4* was also detected in four uterine cancer samples (H1, 3, 4, and 5), three normal uterus samples (G3, 4, and 5), and one ovary sample (G10). Interestingly, the expression of *PAGE4* was also found in two kidney cancer samples (B2 and 5) but not in normal kidney (A1–A11).

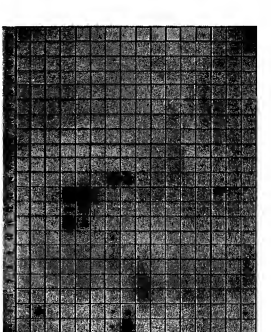

Fig. 1. Dot blot analysis of *PAGE4* probe. Hybridization of *PAGE4* probe on a matched tumor (*T*)/normal (*N*) expression array membrane (Clontech) showed specific hybridization of *PAGE4* in different samples. Samples on the blot are from kidney (*A* and *B*, *1–11*), breast (*D* and *E*, *1–9*), prostate (*D* and *E*, *11–13*), uterus (*G* and *H*, *1–7*), ovary (*G* and *H*, *9–12*), cervix (*G* and *H*, *14*), colon (*J* and *K*, *1–11*), lung (*J* and *K*, *13–15*), stomach (*M* and *N*, *1–8*), rectum (*M* and *N*, *10–16*), and small intestine (*M* and *N*, *19*).

***PAGE4* mRNA Is Expressed in Epithelial Cells of Normal Prostate and Prostate Cancer.** The RNA used for both dot blot and RT-PCR analyses was extracted from whole tissue, which consisted of mixed populations of epithelial cells, smooth muscle cells, and fibroblasts, as well as other cell types. To determine the cell types that express the *PAGE4* mRNA in normal prostate as well as in prostate cancer tissue, we used *in situ* hybridization with biotin-labeled *PAGE4* cDNA as described in "Materials and Methods." As shown in Fig. 2, *PAGE4* mRNA was highly expressed in prostatic epithelial cells of normal prostate (*A*) and in prostate cancer tissue (*B*). There was no detectable signal in cells of the stromal compartment, which suggests that *PAGE4* was specifically expressed in the epithelial cells of the prostate.

***PAGE4* mRNA Encodes for a *M*, 16,000 Cytoplasmic Protein.** We reported previously that the *PAGE4* transcript has a predicted open reading frame of 102 amino acids,

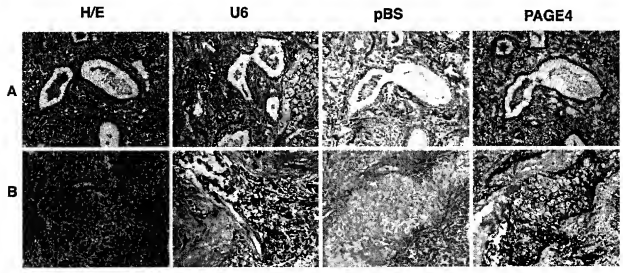

Fig. 2. In situ localization of *PAGE4* mRNA expression. *In situ* hybridization of normal prostate and prostate cancer tissue. *A*, H&E (*H/E*) stains of a representative field of benign prostatic ducts. *PAGE4* is strongly expressed in atrophic basaloid type epithelium. *B*, an example of solid lobular adenocarcinoma, Gleason grade 4 (score 8/10) strongly expressing *PAGE4*. The *PAGE4* signal is comparable in intensity with U6 positive control, in contrast to pBS negative control.

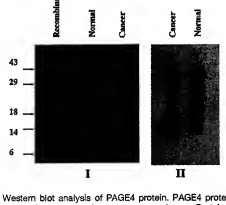

Fig. 3. Western blot analysis of PAGE4 protein. PAGE4 protein is detected in normal prostate and prostate cancer lysate. Protein extracts from normal prostate and prostate cancer (80 μg each) were analyzed on a Tris-tricine 16.5% PAGE and blotted with either antipeptide (*I*) or anti-full-length PAGE4 (*II*) polyclonal antibodies. Purified recombinant PAGE4 (10 ng) protein was used as a positive control.

which can generate a protein of M_r 11,900 in size. To determine the size of PAGE4 protein expressed in normal prostate and in prostate cancer tissue, we generated polyclonal rabbit antibodies against a chemically synthesized PAGE4 peptide as well as a recombinant PAGE4 protein as described in the "Materials and Methods" section. As shown in Fig. 3, a specific band at a M_r of ~16,000 was detected in protein extracts from normal prostate and prostate cancer by both antibodies. The M_r 16,000 kDa band comigrated with the recombinant PAGE4 protein, which indicated that the band detected in normal prostate and in prostate cancer was PAGE4. We did not detect this band using IgG prepared from preimmune rabbit serum or using a brain tissue extract but we did find expression of PAGE4 in placenta which is known to express *PAGE4* mRNA (data not shown).

To determine the location of PAGE4 protein in the cell, we performed two sets of experiments. In the first experiment, we prepared nuclear, cytoplasmic, and membrane fractions from NIH3T3 and PC3 cells stably expressing *PAGE4*. As shown in Fig. 4*A*, a specific M_r 16,000 PAGE4 product, which comigrates with recombinant PAGE4, was detected only in the cytoplasmic fraction of both of the cell lines, and not in the nuclear or membrane fractions. In a second experiment, we transfected 293T cells and infected NIH3T3 cells transiently with an expression (pCI-*PAGE4*) plasmid and performed an immunofluorescence experiment with anti-PAGE4 antibody. As shown in Fig. 4*B*, an intense cytoplasmic staining was observed in both NIH3T3 and 293T cells, which were transfected with *PAGE4* cDNA, indicating that PAGE4 protein is localized in the cytoplasm of the cells. No staining was observed in cells transfected with empty vector (data not shown).

PAGE4-regulated Gene Expression in Infected NIH3T3 and PC3 Cell Lines. Although many CT antigens have now been reported, very little is known about their biological functions. We investigated the ability of *PAGE4* to alter gene expression in the mouse NIH3T3 cell line. NIH3T3 cells do not express *PAGE4* mRNA. We generated several stable cell lines expressing the *PAGE4* transcript and protein; for the analysis, we pooled the RNA from three different stable of lines NIH3T3. To identify the transcript, which could be differentially regulated in the presence or absence of *PAGE4*, we used cDNA microarray hybridization, because it allowed us to screen numerous transcripts simultaneously. The mouse array used in this study contains 2688 cDNAs including known genes and ESTs from unknown genes (Mm-OC-6.1p).[4] Genes that were either up- or down-regulated at least

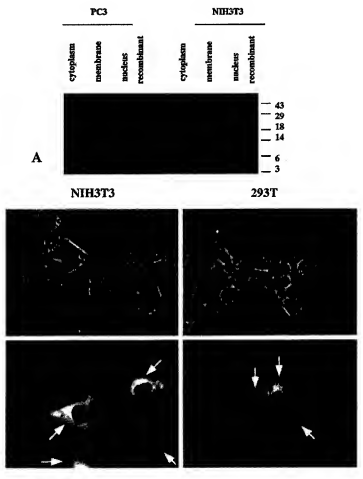

Fig. 4. Detection of PAGE4 protein on transfected cells. *A*, PAGE4 protein is detected in the cytoplasmic fraction of the cell. Protein extracts (cytoplasm, membrane, and nuclear fraction) from *PAGE4*-transfected NIH3T3 and PC3 cell lines were analyzed on a Tris-tricine 16.5% PAGE. Ten μg of protein extract was loaded on each lane. For positive control, 10 ng of recombinant *PAGE4* was loaded. *B*, *PAGE4*-transfected NIH3T3 and 293T cells were fixed, permeabilized using saponin, and incubated with anti-PAGE4 antibodies, followed by an antirabbit IgG-rhodamine conjugate. Intense cytoplasmic staining (*arrows*) was observed in both cell lines.

Table 1 Genes up/down-regulated in PAGE4 and β galactosidase (β Gal) stable cell lines

Gene	NIH3T3		PC3	
	PAGE4	β Gal	PAGE4	β Gal
Dlk1	↑[a]	↑	noc	noc
IGFBP 2	↑	↑	–	–
LpL	↓	–	–	–
SCGN-10[b]	noc	noc	↓	↓

[a] ↑, up-regulated; ↓, down-regulated; –, no change; noc, not on chip.
[b] SCGN-10, uperior cervical ganglia, neural-special 10.

2.5-fold in *PAGE4*-transfected cells, but not in β-galactosidase-transfected cells, were considered for analysis.

Only three genes were found to be altered by 2.5-fold or more (Table 1). One of the genes, that was specifically modulated in *PAGE4*-transfected cells as compared with vector-alone-transfected cells, was *LPL*. LPL is the primary enzyme responsible for the conversion of lipoprotein triglycerides into free fatty acids and monoglycerides. *LPL* was reproducibly down-regulated in *PAGE4*-expressing stable cells (Fig. 5*A*), as compared with vector-alone-transfected or β-galactosidase-transfected cell lines. Two other genes, *Dlk1* and *IGFbp-2*, were up-regulated in *PAGE4*-transfected cells, but they were also up-regulated in β-galactosidase-transfected NIH3T3 cells (Table 1). Thus, this effect of *PAGE4* was not specific. Down-regulation of *LPL* expression in *PAGE4* stable lines was verified by Northern blot analysis. RNA was isolated from each of three independent cell lines that were transfected with either *PAGE4* or empty vector, and that were subjected to Northern analysis using a radiolabeled *LPL* cDNA probe. As shown in Fig. 5*B*, all three of the cell lines stably expressing *PAGE4* had undetectable *LPL* mRNA expression as compared with the cells infected with empty vector or vector expressing the *β-galactosidase* gene.

We then investigated the ability of *PAGE4* to alter gene expression in the PC3 prostate cancer cell line. PC3 is an androgen-independent prostate-cancer cell line and does not express *PAGE4* mRNA.[5] We generated several stable cell lines expressing the PAGE4 transcript and protein, and we analyzed RNA expression with an array that contains 6538 human cDNAs including known genes and ESTs from unknown genes.[4] We found that none of the cDNAs was significantly modulated in the PC3 cells expressing *PAGE4*

[5] Iavarone and Bera, unpublished observations.

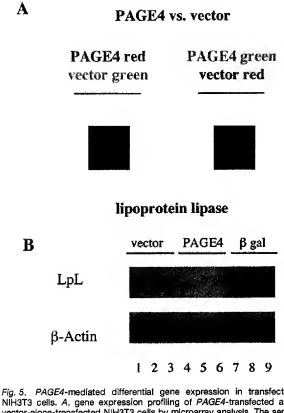

Fig. 5. PAGE4-mediated differential gene expression in transfected NIH3T3 cells. *A*, gene expression profiling of *PAGE4*-transfected and vector-alone-transfected NIH3T3 cells by microarray analysis. The same spot (*LPL*) is detected by two independent experiments in which *PAGE4*-transfected cell RNA is labeled with red or green dye. In *B*, LPL transcript is down-regulated in *PAGE4*-transfected 3T3 cells. An equal amount (2 μg) of polyadenylete RNA from NIH3T3 cells (three independent clones), transfected with either *PAGE4* or vector alone, was size-fractionated and transferred to nitrocellulose filter. Filters were then hybridized with labeled LPL cDNA as described in "Materials and Methods." The filters were stripped and reprobed with labeled actin to determine equal loading of RNA in the membrane.

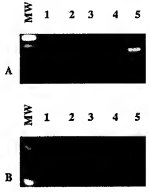

Fig. 6. RT-PCR and PCR analyses of *LPL* gene in PC3 cell line. Ethidium bromide-stained 1.2% agarose gels: in *A*, *LPL* cDNA was amplified with specific primers for the 3' untranslated region of *LPL* mRNA. A specific band was amplified by using normal prostate cDNA (Clontech; *Lane 2*), PC3 genomic DNA (*Lane 4*) and human genomic DNA (*Lane 5*). No band was amplified without template (*Lane 1*) and using PC3 cDNA (*Lane 3*). In *B*, integrity of each mRNA and genomic DNA samples used was tested by amplifying the *actin* gene using specific primers for actin.

including LPL, which was down-regulated in mouse array analysis (see below).

Discussion

PAGE4 is a CT antigen expressed in normal prostate, testis, and placenta, and highly expressed in prostate and uterine cancer. In this report, we demonstrate that *PAGE4* mRNA expression is restricted to the epithelial cells of normal prostate and prostate carcinomas. The PAGE4 protein is *M*, 16,000 in size and is localized in the cytoplasmic compartment of the cell. To investigate the function of *PAGE4*, we introduced *PAGE4* into a mouse fibroblast cell line (NIH3T3) and also into a prostate cancer cell line (PC3) and found that LPL is down-regulated in NIH3T3 cells expressing *PAGE4*, but not in PC3 cells.

Expression of PAGE4 Affects *LPL* Gene Expression. LPL, a rate-limiting enzyme responsible for the hydrolysis of circulating triglyceride, is bound to the luminal surface of capillary endothelium in adipose tissue and muscle (10). Although LPL is functional at the surface of the endothelial cells, it is not clear which cells synthesize the enzyme and what the cellular distribution of LPL is within the tissue. It has been reported that the expression of the *LPL* gene is regulated by progesterone and protein kinase A in undifferentiated hepatoma cells (11). Reduction in LPL activity has been implicated in cachexia in cancer patients. Cachexia is a common feature of malignant disease and is characterized by marked weight loss, anorexia, and extensive breakdown of body fat and skeletal protein because of homeostatic disturbances (12). It has been reported that the *LPL* gene in humans is localized on chromosome 8p22 (13), and there is a frequent allelic loss of chromosome 8p22 loci in human prostate cancer. Because we observed down-regulation of LPL in NIH3T3 cells expressing *PAGE4*, but not in the prostate cancer cell line PC3, we investigated whether the *LPL* gene is expressed in the PC3 cell line. As shown in Fig. 6*A*, *Lane 3*, the *LPL* gene is not expressed in the PC3 cell line, thus, *PAGE4* could not possibly down-regulate LPL expression in this cell line. To determine whether the *LPL* gene is deleted in PC3 cells, we did a PCR analysis on chromosomal DNA isolated from PC3 cells. As shown in Fig. 6*A*, *Lane 4*, the intensity of the PCR product from PC3 DNA is one-half the intensity of the PCR product (Fig. 6*A*, *Lane 5*) obtained from normal human chromosomal DNA, which suggests that at least one of the *LPL* alleles is deleted in PC3 cells. Further analysis of the PC3 cell line is needed to establish the exact copy number of the *LPL* gene in PC3 cells, and the significance of the LPL deletion in prostate cancer and its down-regulation by *PAGE4* gene needs further study.

Although CT antigens have been identified for over a decade, no function has been described for these antigens in the literature. These antigens are encoded quite frequently by genes located on the X chromosome. There is speculation that some CT gene products are transcriptional factors, but there is no direct experimental evidence supporting that concept. Two of the previously reported CT antigens, SCP-1 and CT9 have small basic domains and several conserved motifs, which are characteristics of DNA-binding proteins

Fig. 7. Amino acid analysis of PAGE4. Amino acid sequence analysis of PAGE4 was performed using the PROSITE program. *Underlined*, two potential protein kinase C phosphorylation sites (amino acids 2–4 and 73–75) contained in PAGE4 protein. *Boxed*, two potential casein kinase 2 phosphorylation sites (amino acids 30–33 and 71–74).

(14, 15). Amino acid sequence analysis of *PAGE4* showed no such motifs in the PAGE4 protein.

At this point, it is not clear whether the regulatory effect of *PAGE4* on the *LPL* gene is direct or indirect. Because PAGE4 protein is localized in the cytoplasm of the cell, it is more likely that *PAGE4* indirectly regulates the expression of the *LPL* gene. It is also possible that *PAGE4* is posttranslationally modified and involved in a signal transduction pathway. Analysis of the *PAGE4* amino acid sequence using the PROSITE program of the Swiss Institute of Bioinformatics ExPASy proteomics server[6] (16, 17) predicts several potential phosphorylation sites. As shown in Fig. 7 there are two casein kinase 2 (SQOE and TRSE) sites and two protein kinase C phosphorylation sites (SAR and SER). The significance of these phosphorylation sites, as well as the mechanism by which *PAGE4* alters the expression of the *LPL* gene, is yet to be determined. To our knowledge, this is the first report of CT antigen-mediated gene regulation in the literature.

PAGE4, a CT Antigen, Is a Potential Target for Cancer Vaccines. CT antigens are a distinct class of antigens that have a restricted pattern of expression in essential tissue and aberrant expression in many different tumor types (18). CT antigens are often expressed at higher levels in testis and placenta, which are known to express only low amounts of MHC class I molecules. Thus, expression of CT antigens in these normal tissues should not lead to T-cell activation and this makes these antigens attractive candidates for cancer vaccines (19). Our earlier studies demonstrated that *PAGE4* is expressed in prostate and uterine cancer. Data presented here indicate that *PAGE4* is also expressed in cervical, ovarian, and kidney cancer and increases its attractiveness as a cancer vaccine target.

Acknowledgments

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[6] Internet address: http://www.expasy.ch.

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